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Marek's disease virus unique genes pp38 and pp24 are essential for transactivating the bi-directional promoters for the 1.8 kb mRNA transcripts

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Abstract The *pp38* and *pp24* genes of Marek's diseases virus (MDV) share the same promoter, which controls the transcription of pp38 or pp24 and a 1.8-kb mRNA bi-directionally. To understand the trans-activating activity of pp38 and pp24 on the bi-directional promoter, both genes were cloned into pcDNA-3 or pBudCE4.1 vectors either singly or in combination. These plasmids were expressed in transfected chicken embryonic fibroblast (CEF) cells. Chloramphenicol acetyltransferase (CAT) activity expressed under the control of the promoter in CEF co-transfected with pP(1.8 kb)-CAT and pBud-pp38-pp24 was significantly higher than that following transfection with only pBud-pp38 or pBud-pp24. This indicates the combination of pp24 and pp38 together are essential for the activation of the promoter. In DNA mobility shift assays, the promoter binds to pp38 and pp24 together, but not to pp38 or pp24 alone. By competitive inhibition tests with a set of DNA fragments from the promoter region, the sequence 5′-CTGCTCATTT-3′ was identified as the core sequence for binding by pp38-pp24 in up-regulation of the bi-directional promoter activity.

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Introduction

Marek's disease (MD) is caused by serotype 1 Marek's disease virus (MDV-1), a member of the alpha-herpesviruses. MDV-1 can induce tumors characterized by the formation of T-cell lymphomas in various visceral organs and tissues in the infected chickens. These virally induced tumors can be effectively controlled by vaccination with all three serotypes of MDV. The pathology of MDV has been studied extensively but the molecular basis for neoplastic transformation of lymphocytes by MDV remains to be fully investigated.

Based on molecular virology studies, four genes of MDV-1 have been shown to be related to the tumorogenecity of MD: the 1.8-kb transcript with 132-bp repeats [1, 2], the 38 kD phosphorylated protein (*pp38*) [3–5], *meq* [6], and *ICP4* [7]. Among these genes, the *meq* gene has been studied most extensively. The N-terminal bZIP domain of *Meq* is similar to *jun/fos* oncogene family and the proline-rich C-terminal contains a transactivation domain. Over-expression of *Meq* leads to transformation of a rodent fibroblast cell line, Rat-2, and protects it from apoptosis [8]. The wild-type *Meq* transactivates its own promoter, but represses the transcription of the *pp38/pp24* divergent promoter and of *ICP4* promoter [9, 10]. In recent studies, using MDV cosmids to generate a mutation, *Meq* was shown to be an essential gene for tumor induction [11]. In addition to *Meq*, the *ICP4* and *pp38* were also shown to be involved in maintenance of the transformation of MD cell lines, e.g., MSB1 cells [7].

The pp38 protein contains MDV-specific antigens detected in both lytically infected cells and MD tumor cell lines, and it has been speculated that pp38 is involved in MDV-transformation since its identification [3, 4, 12]. However, the pp38 gene is also expressed by the non-oncogenic MDV-1 vaccine strain CVI988/Rispens [7, 13, 14] and therefore it is unlikely to be involved in transformation [5, 15, 16]. In a recent study, using MDV cosmids to generate a mutation, the pp38 gene was definitively shown to be involved in early cytolytic infection in lymphoid organs but not in the induction of tumors [17]. Other reports have indicated that pp38 may have a role in immunosuppression induced by MDV [1, 14, 18, 19].

A 1.8-kb mRNA transcript is present in oncogenic viruses but is truncated in the attenuated variants [20]. This transcript is mapped in the internal repeat long (IRL) region of the MDV genome containing the tandem repeats of the 132-bp repeat region. This repeat region is expanded during attenuation by in vitro passage and also found in vaccine strain CVI988 [21, 22]. Recently, it was shown that the expansion of the 132-bp repeat is not sufficient in itself to attenuate pathogenic MDV [23].

Between *pp38* and the 1.8-kb mRNA family in the IR_L region of the MDV genome contains, a bi-directional transcriptional promoter sequence that controls the transcription of both genes in opposite orientations. Although the promoter sequence is only 305 bp in size [24], it contains the replication origin and several *cis*-acting motifs such as TATA-box, CAAT-box, Oct-1, and Sp1 [2, 25, 26]. There is a 90-bp putative replication origin of the MDV genome in the middle of this promoter region [2, 27] which shares more than 80% nucleotide identity among the three serotypes of MDV, and over 70% identity with those of other α-herpesviruses [28]. A bi-directional transcriptional promoter sequence also exists between the *pp24* gene and the 1.8-kb mRNA family on TR_L region of the MDV genome [29].

We have been interested in the role of pp38 in transactivating the bi-directional promoter. We have previously shown that CAT activity under the control of the bi-directional promoter was significantly lower in the pp38 deletion virus, rMd5/Δpp38-, than in the parental rMd5 virus in infected CEF. This suggests that pp38 may be a factor involving in regulation of the promoter. In addition, a DNA mobility shift assay showed a 73-bp fragment of the bi-directional promoter binds to parental rMd5-CEF or rMd5/Δpp38-CEF transfected with pcDNA-pp38, but not rMd5/Δpp38-CEF alone or CEF transfected with pcDNA-pp38. This indicates that pp38 may be necessary for regulating the bi-directional promoter [30]. In a separate study, we co-transfected CEF with both pcDNA-pp38 and pcDNA-pp24 expressing plasmid DNA and showed that the expression of green fluorescence protein gene (*EGFP*) was under the control of the bi-directional promoter [31]. The present study demonstrates the transactivation activity of both pp38 and pp24 together on the bi-directional promoter and identifies a seven base binding motif using the gel mobility shift assay.

Materials and methods

Construction of recombinant plasmids

A recombinant plasmid pP(1.8 kb)-CAT was constructed in a previous study according to published procedure [30]. To construct pBud-pp38-pp24 for co-expression of both pp38 and pp24, the complete pp38 gene was amplified from MDV GA strain and cloned into pBudCE4.1 vector (Invitrogen) at *Bam*HI and S*al*I sites, and a recombinant plasmid pBud-pp38 was selected. The complete pp24 gene was amplified from MDV strain GA and cloned into pBudCE4.1 and pBud-pp38 at *Kpn*I and *Xho*I sites respectively. Then recombinants pBud-pp24 and pBud-pp38-pp24 were selected. In plasmid pBud-pp38-pp24, the pp38 and pp24 were expressed under the control of promoters P_{CMV} and $P_{EF-1\alpha}$ in the expressing vector pBudCE4.1, respectively. Two additional plasmids, pcDNA-pp38 and pcDNA-pp24, were constructed by using the pcDNA-3.1/Zeo(+) vector (Invitrogen) as described previously [30, 31]. All of the above plasmids were purified with a QIA prep Spin Miniprep Kit (Qiagen).

Transfection of CEF with plasmid DNA

Secondary CEF monolayers were prepared in 35 mm plates and transfection was carried out when CEF monolayers were about 70% confluent using the LipofectAMINETM reagent (Gibco BRL) according to the manufacturer's instructions. For each transfection, we used 2 μg of plasmid DNA and 4 μl of LipofectAMINETM reagent (Gibco BRL), and the transfection was stopped after 8 h. Detailed procedures are given in previous reports [30, 31].

Determination of CAT activity in transfected CEF

Two days after transfection with plasmid pP(1.8 kb)-CAT or co-transfection with plasmids pP(1.8 kb)-CAT and other pp38- and/or pp24-expressing plasmids, the transfected CEF were harvested and resuspended in 500 μl lysis buffer (0.25 M Tris–Hcl, pH = 7.0) per 35 mm plate. The collected cells were lysed and the CAT activity was measured as reported [30]. Six to eight replicates of transfection were carried out for each group. The significance of the differences among groups was analyzed by Student's *t*-test.

Preparation of cytoplasmic extracts for DNA-binding assay

CEF monolayers in 60 mm dishes were transfected with plasmids pBud-pp38-pp24, pcDNA-pp38, and pcDNA-pp24 alone or co-transfected with pcDNA-pp38. The culture medium was removed about 48 h after transfection and washed 3 times with PBS. The cytoplasmic extracts of the transfected cells were prepared as previously [30]. Then 40 µl samples were aliquoted into tubes and stored at –80°C.

Preparation of Digoxigenin-labeled DNA probe for mobility shift assay

The Digoxigenin- (Dig-) labeled DNA probe contained a 73 bp fragment corresponding to subregion ll(from base –514 to –442 of *pp38* ORF) of the bi-directional promoter (Fig. 1) and was prepared as described previously [30]. The procedure for DNA mobility shift assay and detection of retarded DNA probe were conducted as previously reported [30].

The detection of pp38 bound to retarded DNA

To identify the specific protein of pp38 in the retarded band, a pp38-specific monoclonal antibody (Mab) H19 was used in a Western blot. A piece of nitrocellulose (NC) membrane was put into the blocking buffer (10% skimmed milk) overnight at 4°C. Afterwards, it was washed with larger volumes of PBST 3 times, and incubated in the blocking buffer containing Mab H19 (1:1,000) at 37°C for 1 h. After washing 3 times with PBST, the membrane was transferred to the blocking buffer containing HRP conjugated anti-mouse IgG (Promega, Cat. W4021), and incubated for 1 h at 37°C. The NC membrane was washed as above, and placed in the DAB buffer (15 ml PBS, 20 µl H_2O_2, 9 mg DAB) to demonstrate color. The color reaction was stopped by 2 M H_2SO_4.

Competitive inhibitory test in DNA mobility shift assay

Based on the previous study, the 73-bp fragment of subregion ll(from base –514 to –442) in the promoter was the only fragment retarded by pp38-associated proteins from MDV-CEF or pp38 and pp24 together from transfected CEF [30, 31]. To further identify the precise sequence bound by pp38-pp24, double-stranded DNA samples of small overlapping subfragments covering the 73-bp fragment (#1: –514 to –490; #2: –500 to –457; #3: –467 to –442) were synthesized by a commercial service (Takara Biotechnology (Dalian) Co., Ltd). These were dissolved into TE (10 mmol/l Tris.Cl; 1 mmol/l EDTA, pH = 8.0) buffer to 100 µmol/l, respectively. The total volume of competitive inhibitory reaction buffer was 80 µl, including: 10 µl of subfragments; 10 µl 73 bp Dig-labeled probe DNA (20 µmol/l) [30]; 40 µl of pBud-pp38-pp24 transfected cytoplasmic extracts; 8 µl 0.1%BSA; and 12 µl distilled water. The components were thoroughly mixed and kept at 30°C for 30 min or at 4°C over night. Then these samples were applied to a nondenaturing 4% polyacrylamide gel for electrophoresis. The gel was transferred to a piece of NC membrane for immunological detection and, the procedure was similar to a DNA mobility shift assay previously reported [30]. The specific subfragment was identified according to the color reaction. Based on the subfragment that competitively inhibited the retardation of the Dig-labeled 73-bp fragment, three further smaller fragments were synthesized accordingly and used in the

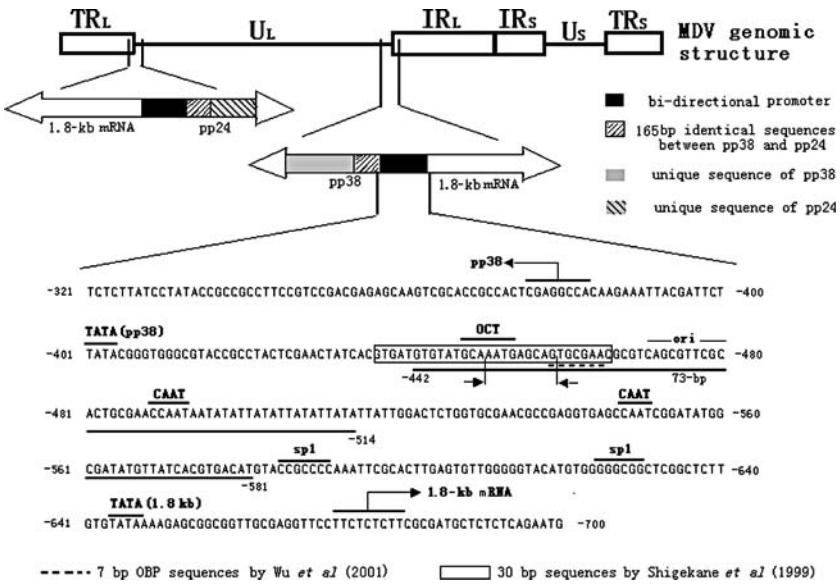

Fig. 1 The structure of the bi-directional promoter and locations of *pp38/pp24* and 1.8-kb mRNA genes on MDV genome. The sequence number is based on a previous publication (Cui et al. [4]). The sequence between two black arrows is the motif for binding by pp38/pp24

next series of competitive inhibitory tests. These experiments were repeated 4–5 times until the precise protein factor(s)-bound DNA sequence was determined.

Results

The pp38 and pp24 together transactivate the bi-directional promoter for CAT expression

In experiment 1, we compare the CAT activity transfected with pp38 and pp24 cloned into the pcDNA3.0 vector. As shown in (1) of Table 1, without substrate enhancer, the CAT activity under the control of the bi-directional promoter in CEF transfected with pP(1.8 kb)-CAT alone (3.8 pg/ml) was at the same level as the negative control pCAT-basic (3 pg/ml) without promoter. Co-transfection of pP(1.8 kb)-CAT with only pcDNA-pp38 (4 pg/ml) or pcDNA-pp24 (3.8 pg/ml) in CEF did not increase the level of CAT activity. Co-transfection of pP(1.8 kb)-CAT with both pcDNA-pp38 and pcDNA-pp24 together also did not significantly increase the CAT activity (5.2 pg/ml). However, when substrate enhancer was added as in (2) of Table 1, co-transfection of pP(1.8 kb)-CAT with both pcDNA-pp38 and pcDNA-pp24 did significantly increase the CAT activity (230.9 pg/ml) ($P < 0.05$). In experiment 2, we cloned pp38 or pp24 into pBud vector. As shown in Table 2, a significant increase in CAT activity was obtained in co-transfection with pBud-pp38-pp24, but not with pBud-pp38 or pBud-pp24 or pBud-pp38 + pBud-pp24. The result of these two experiments indicates that co-expression of pp38 and pp24 was essential to up-regulate the transcriptional activity of the bi-directional promoter for *CAT* gene expression, but co-transfection of pp38 or pp24 alone was ineffective.

The 73-bp Dig-DNA probe was retarded by cellular extracts from CEF co-expressing both pp38 and pp24

A DNA mobility shift assay was carried out by gel electrophoresis on cellular extracts of CEF transfected with different plasmids and a Dig-labeled 73-bp DNA probe was used in Western blot. As shown in Fig. 2, upper panel, the 73-bp Dig-DNA probe was retarded only by cellular extracts from CEF co-transfected with both pcDNA-pp38 and pcDNA-pp24 (1A, Lane 4 and 3A, Lane 4) or transfected with pBud-pp38-pp24 (2A, Lane 2 and 3A, Lane 3). However, cellular extracts from CEF transfected with pcDNA-pp38 or pcDNA-pp24 alone in three independent assays did not induce retardation of the Dig-DNA probe in the same assays. It suggests that both pp38 and pp24 together are essential for binding the 73-bp DNA fragment of the bi-directional promoter, but pp38 or pp24 alone was not effective.

Table 1 Comparison of the CAT concentrations expressed in CEF transfected with pP(1.8 kb)-CAT or co-transfected with other pp38/pp24-expressing plasmids (Experiment 1)

Transfected plasmids	pCAT-Basic	pP(1.8 kb)-CAT	pP(1.8 kb)-CAT + pcDNA-pp38	pP(1.8 kb)-CAT + pcDNA-pp24	pP(1.8 kb)-CAT + pcDNA-pp38 + pcDNA-pp24
CAT (pg/ml)					
(1)	3.0 ($n = 6$)	3.8 ± 0.90 (3.0–5.0, $n = 6$)	4.0 ± 0.58 (3.0–5.0, n = 6)	3.8 ± 0.69 (3.0–5.0, $n = 6$)	5.2 ± 0.97 (4.0–6.0, $n = 6$)
(2)	167.6 ± 4.8 (159–175) $n = 8$)	170.5 ± 7.1 (159–182, $n = 8$)	179.5 ± 14.0 (162–202, $n = 8$)	178.8 ± 10.6 (159–189, $n = 8$)	230.9 ± 20.97 (177–252, $n = 8$)

CAT concentration was measured 48 h after transfection as described in Materials and methods. The numbers are: mean ± standard errors (n = number of transfection). (1) values measured when no substrate enhancer was added. (2) values measured after substrate enhancer was added. Co-transfection of pP(1.8 kb)-CAT, with either pcDNA-pp38 or pcDNA-pp24 did not increase CAT activity significantly as compared to control [pCAT-Basic or pP(1.8 kb)-CAT] ($P > 0.05$). Co-transfection of pP(1.8 kb)-CAT with both pcDNA-pp38 and pcDNA-24 also did not increase CAT activity significantly when no substrate enhancer was added ($P > 0.05$) in (1), but it increased CAT activity significantly ($P < 0.05$) with substrate enhancer in (2)

Table 2 Comparison of the CAT concentrations expressed in CEF transfected with pP(1.8 kb)-CAT or co-transfected with other pp38/pp24-expressing plasmids (Experiment II)

Transfected plasmids	pCAT-Basic	pP(1.8 kb)-CAT	pP(1.8 kb)-CAT + pBud-pp38	pP(1.8 kb)-CAT + pBud-pp24	pP(1.8 kb)-CAT +pBud-pp38 + pBud-pp24	pP(1.8 kb)-CAT +pBud-pp38-pp24
CAT (pg/ml)						
(1)	4.0 (n = 6)	4.7 ± 0.87 (4.0–6.0, n = 6)	5.0 ± 0.76 (4.0–6.0, n = 6)	4.8 ± 0.64 (4.0–6.0, n = 6)	5.67 ± 0.82 (4.0–6.0, n = 6)	17.2 ± 0.3.44 (13.0–24.0, n = 6)
(2)	209.5 ± 6.1 (199–219, n = 8)	239.8.3 ± 12.8 (218–256, n = 6)	250.8 ± 15.9 (225–270, n = 6)	248.2 ± 13.4 (223–262, n = 6)	289.5 ± 26.8 (260–320, n = 6)	653.2 ± 40.5 (577–703, n = 6)

CAT concentration was measured 48 h after transfection as described in Materials and methods. The numbers were: mean ± standard errors (n = number of transfection). (1) values measured when no substrate enhancer was added; (2) values measured after substrate enhancer was added. Co-transfection of pP(1.8 kb)-CAT with pBud-pp38-pp24 increased CAT activity significantly ($P < 0.01$) whereas with both pBud-pp38 and pBud-pp24 did not

To demonstrate the retarded 73-bp DNA fragment of the bi-directional promoter is bound by the pp38-pp24 complex, we used monoclonal antibody H19 specific for detecting pp38 in a Western blot. As shown in Fig. 2, bottom panel, only samples treated with cellular extracts from CEF transfected with pBud-pp38-pp24 (2B, Lane 2 and 3B, Lane 3) or co-transfected with both pcDNA-pp38 and pcDNA-pp24 (1B, lane 4; 2B, Lane 3 and 3B, Lane 4) showed pp38 specific Mab H19 recognized bands at the positions of retarded Dig-73bp probe. In addition, Mab H19 also recognized the unbound pp38-pp24 complex bands, which migrated faster than bands from the retarded Dig-73 bp. In the lanes receiving the cellular extracts of CEF transfected with pcDNA-pp38 alone, Mab H19 recognized the free pp38 bands (1B, Lane 3; 2B, Lane 4 and 3B, Lane 1) that had migrated further down the gel than bands from the unbound pp38-pp24 complex. In samples treated with cellular extracts of CEF transfected with pcDNA-pp24 alone, no protein band was detected as expected (1B, Lane 2; 2B, Lane 5; and 3B, Lane 2).

Determination of the sequence on the bi-directional promoter bound by pp38-pp24 complex

Competitive inhibition tests were carried out to determine the precise sequence on the promoter bound by the pp38-24 complex using a DNA mobility shift assay. In this test, the 73-bp Dig-DNA probe was treated with the mixtures of cellular extracts from CEF transfected with pBud-pp38-pp24 and unlabeled subfragments of the probe DNA as competitors for binding pp38/pp24. The tests were carried out in four steps: A, B, C and D. In each step, the competitive DNA subfragment was smaller in size. As shown in Fig. 3A, the subfragment from base –467 to –442 (Lane 3) inhibited the retardation of the 73-bp Dig-DNA probe. In Fig. 3B, the subfragment from –462 to –447 (Lane 2) inhibited the retardation. In Fig. 3C, the subfragment from –459 to –450 (Lane 3) inhibited the retardation. In Fig. 3D, all four overlapped subfragments from –460 to –452 (Lane 1), –459 to –451 (Lane 2), –458 to –450 (Lane 3), and –457 to –449 (Lane 4) showed competitive inhibitory effect at different levels. Among them, the subfragment from –459 to –451 showed almost complete inhibition (Lane 2). These results suggest that the sequence 5′-CTGCTCATTT-3′, from base –459 to –450 may be the core sequence for binding the pp38-pp24 complex.

Discussion

The *pp38* and *pp24* MDV genes are unique and have no homologs with other groups of herpesviruses or with any other genomes thus far identified and sequenced [4]. The

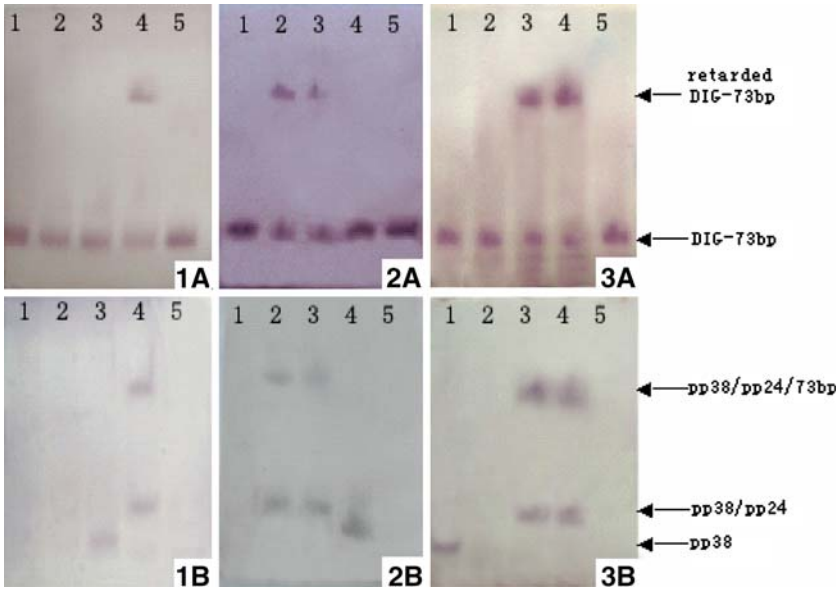

Fig. 2 DNA mobility shift assays of a Dig-labeled 73-bp fragment treated with cellular extracts of CEF transfected with different plasmids. Three independent assays designated as 1, 2, and 3 were conducted with different lysate samples. Each assay had two duplicates, A and B. Panel **A** shows the retarded protein-bound Dig-DNA probe. Panel **B** shows pp38 is bound to the retarded DNA fragments using Mab H19 specific for p38. In assay **1A** and **1B**: Lane 1 (normal CEF); Lane 2 (CEF transfected with pcDNA-pp24); Lane 3 (CEF transfected with pcDNA-pp38; Lane 4 (CEF co-transfected with pcDNA-pp24 and pcDNA-pp38); Lane 5 (free 73-bp Dig-DNA probe). In assay **2A** and **2B**: Lane 1 (normal CEF); Lane 2 (CEF transfected with pBud-pp38-pp24); Lane 3 (CEF co-transfected with pcDNA-pp24 and pcDNA-pp38); Lane 4 (CEF transfected with pcDNA-pp38); or Lane 5 (pcDNA-pp24). In assays **3A** and **3B**: Lane 1 (CEF transfected with pcDNA-pp38); Lane 2 (pcDNA-pp24); Lane 3 (CEF transfected with pBud-pp38-pp24); Lane 4 (CEF co-transfected with pcDNA-pp24 and pcDNA-pp38); Lane 5 (free 73-bp Dig-DNA probe)

pp38 gene is located at the junction of IRL and U_L and the *pp24* gene is located at the junction of TR_L and U_L of the MDV genome. Both genes have the identical upstream bi-directional promoter sequences between them and the 1.8-kb mRNA transcripts. Also, they share the same N-terminal 55 amino acids [29]. However, both pp38 and pp24 proteins were detected in immunoprecipitation of MDV infected CEF with Mab H19 [32], but only pp38 was detected in a Western blot. Since pp24 was not detected by Mab H19 in a denatured condition in a Western blot, and also did not have a Mab H19 epitope, we concluded it may be associated with pp38 as a complex [25].

A bi-directional promoter of about 300 bp is located between the transcription start sites of the *pp38* or *pp24* gene and the 1.8-kb mRNA transcript [2, 4]. Within the promoter, there are two TATA boxes for gene transcription, and several enhancer motifs including Sp1, Oct1, and CAAT. In addition, a DNA replication origin and 17-bp reverse repeats were located within this region [27]. Shigekane et al. [24] reported that the bi-directional promoter activity was regulated by a viral or cellular factor(s) induced by MDV infection. This factor(s) bind a 30 bp fragment in the promoter region. It is not known which viral product was involved in transactivating this promoter. Our previous study showed CAT activity was significantly lower in a pp38 deletion virus than in the parental rMd5 virus. Co-transfection of rMd5Δpp38 with pcDNA-pp38 restored the CAT activity but not to the same level as the parental rMd5 virus. This suggested that pp38 plays an important role in regulating the transcriptional activity of the bi-directional promoter, but that an additional factor may also be necessary [30].

The experiments in this article support our previous conclusions. We found pp24 is necessary in addition to pp38 for transcriptional activity of the bi-directional promoter. In our co-transfection experiments, we found both pp38 and pp24 expression is necessary for activating the promoter for CAT expression using pBud-pp38-pp24. Neither pBud-pp38 (pcDNA-pp38) nor pBud-pp24 (pcDNA-pp24) alone was able to activate the promoter. Co-transfection with both pcDNA-pp38 and pcDNA-pp24 increased CAT expression significantly but not with pBud-pp38 and pBud-pp24 combination. This may be due to the promoter strength within the vector itself. However, when both genes were cloned into the pBud vector (pBud-pp38-pp24), it was significantly more efficient in activating the CAT expression than pBud-pp38 and pBud-pp24 combination. This may be due to the efficiency of transfection with two plasmid DNAs instead of three into the same cell. We have previously shown that co-transfection of CEF with pp38 and pp24 activates a 1.8 kb-EGFP expressions in an immunofluorescence test with anti-pp38 Mab H19.

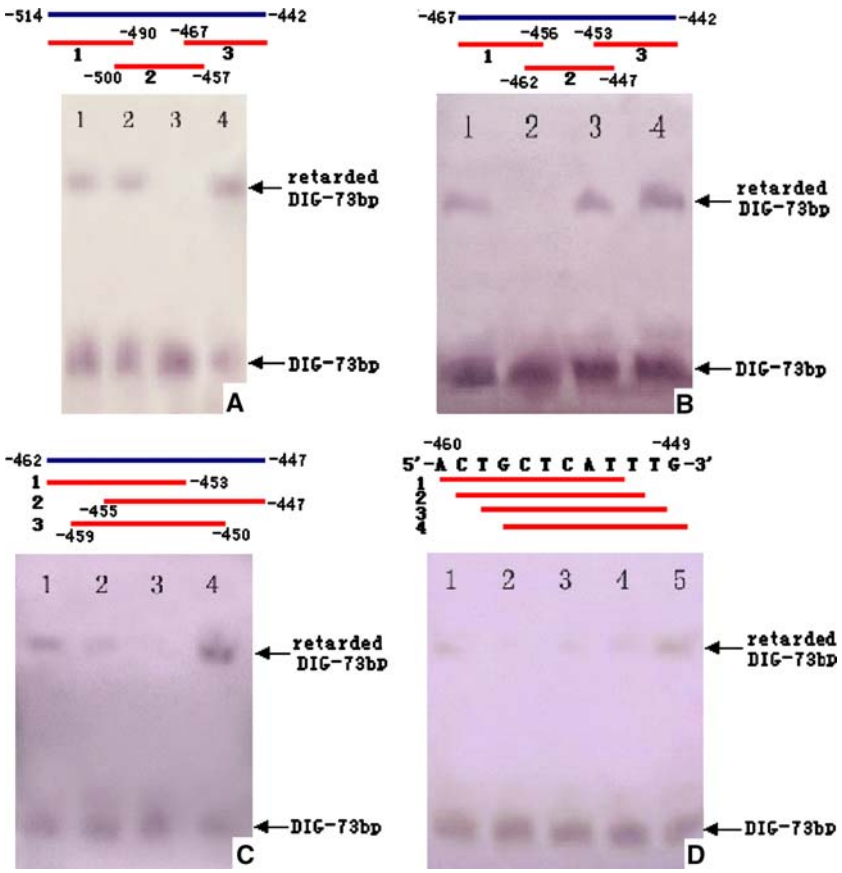

Fig. 3 Competitive inhibition tests in DNA mobility shift assays to determine the exact sequence on the promoter bound by pp38/pp24 complex. The tests were carried out in 4 successive steps of smaller competitive DNA subfragments (Panels **A**, **B**, **C,** and **D**). Panel **A**: Lane 1 (–514 to –490); Lane 2 (–500 to –457); Lane 3 (–467 to –442); Lane 4 (no competitor DNA). Lane 3 showed the inhibition of retardation. Panel **B**: Lane 1 (–467 to –456); Lane 2 (–462 to –447); Lane 3 (–453 to –442); Lane 4 (no competitor DNA). Only Lane 2 showed the inhibition of retardation. Panel **C**: Lane 1 (–462 to –453); Lane 2 (–455 to –447); Lane 3 (–459 to –450); Lane 4 (no competitor DNA). Only Lane 3 showed complete inhibition of retardation. Panel **D**: Lane 1 (–460 to –452); Lane 2 (–459 to –451); Lane 3 (–458 to –450); Lane 4 (–457 to –449); Lane 5 (no competitor DNA). Lane 2 almost completely inhibited retardation of the 73-bp probe. Lanes 1, 3, and 4 also showed partial inhibitory effects

The expression of pp38 and pp24 with 1.8 kb-EGFP was co-localized in the same cell as shown by two different colors with Rodamine and FITC. No expression was detected in cells co-transfected with either pp38 or pp24 alone [31]. These results taken together indicate that co-expression of pp38 and pp24 transactivates the bi-directional promoter resulting in expression of CAT or the EGFP, but transactivation does not occur with expression of pp38 or pp24 alone. In the panel B of Fig. 2, the pp38-specific Mab H19-recognized a free protein band following pcDNA-pp38 transfection, and bands from co-transfection with pBud-pp38-pp24 or co-transfection with pcDNA-pp38 and pcDNA-pp24 combination. This may be additional evidence that pp38 and pp24 form a complex.

Using competitive inhibition tests in DNA mobility shift assays with the 73-bp Dig-DNA probe of the promoter we have identified the sequence 5-CTGCTCATTT-3′ from base –459 to –450 as the core sequence for binding by pp38-pp24 complex. Interestingly, this sequence is located within the 30 bp of an enhancer motif of the promoter reported by Shigekane et al. [28]. Our previous study indicated that the transcriptional activity of the bi-directional promotor was significantly higher for the 1.8 kb mRNA than for the pp38 [30]. It is possible that this binding may be involved in enhancing the 1.8 kb mRNA transcripts but it requires further experimentation. We are planning to perform Northern blot experiments with rMd5Δpp38 and its revertant virus to verify the enhancement in vivo.

In a published study using pp38 deletion mutant, rMd5Δpp38, the pp38 was shown to be involved in early cytolytic infection in lymphocytes but not in the induction of tumors [11]. Interestingly, the core sequence recognized by pp38/pp24 complex is adjacent to a replication origin [27], and is connected directly with a 7-bp sequence TTCGCAC, a motif bound by origin-binding protein [33].

It is therefore reasonable to speculate that the pp38-pp24 binding complex may be involved in MDV replication.

In this article, we demonstrated that the pp38/pp24 complex binds to the bi-directional promoter and transactivates 1.8 kb-mRNA. It is interesting to speculate how cytoplasmic proteins like pp38/pp24 could bind to a part of the naked herpesvirus genome, which should be solely in the nucleus. The MDV genome contains numerous unique genes not present in other herpesvirus and we have no knowledge on the regulation of these unique genes and their interactions at the present time. Based on the present finding, we speculate that the transactivation of the promoter activity may be solely in the cytoplasm. It is also possible that a small amount of expressed pp38 and pp24 is transported to the nucleus and this may be sufficient to activate the promoter activity. Our present finding on the role of pp38 and pp24 in transactivating the bi-directional promoter hopefully will stimulate further research toward understanding MDV replication at the molecular level.

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